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A flux-sensing mechanism could regulate the switch between respiration and fermentation

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Rate of sugar uptake determines the global metabolic phenotype of yeast

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Abstract

The yeast *S. cerevisiae* can show different metabolic phenotypes (e.g. fermentation and respiration). Based on data from the literature, we argue that the substrate uptake rate is the core variable in the system that controls the global metabolic phenotype. Consequently the metabolic phenotype that the cell expresses is not dependent on the type of the sugar or its concentration, but only on the rate at which the sugar enters the cell. As this requires the cells to 'measure' metabolic flux, we discuss the existing hints towards flux-sensing mechanism in this organism and also outline several aspects of the respectively involved flux-dependent regulation system. It becomes clear that the sensing and regulation system that divides the taken up carbon flux into the respiratory or fermentative pathways is complex with many molecular components interacting on multiple levels. To obtain a true understanding about how the global metabolic phenotype of *S. cerevisiae* is being realized, different tools and approaches from systems biology will be required.

Introduction

Microorganisms are constantly facing changing environments, for example, in terms of their nutrient availability. In order to be informed about the characteristics of the environment, sensory systems are required. Classically, these are either transmembrane receptors (Holsbeeks *et al.*, 2004; Rubio-Texeira *et al.*, 2010) or intracellular receptors (such as transcription factors) whose activity is modulated by

their specific ligands. While for certain substrate molecules we know such sensory systems (in yeast e.g. the transmembrane receptors Snf3p and Rgt2p (Johnston & Kim, 2005), or the intracellular receptor Gal3p (Sellick & Reece, 2005; Campbell *et al.*, 2008), for many other substrates we have not yet identified specific sensors. The question is whether we simply do not know them yet or whether cells recognize these metabolites in a different way.

An alternative way to sense the presence of a certain carbon source would be by measuring the metabolic flux that derives from its degradation. In this way cells could perceive what substrate is being imported and at which rate. However, all sensing mechanisms in biology are based on concentration measurements, so cells would need to translate a rate (i.e. flux) into concentrations of certain flux-signaling molecules. Actually, it has been shown for *E. coli* in a synthetic, engineered system that it is possible for cells to 'measure metabolic fluxes' and use them for regulation (Fung *et al.*, 2005). In addition, a recent computational study that modeled *E. coli* central metabolism suggested that intracellular metabolic fluxes is indeed used to indirectly perceive the presence of a particular carbon source and the rate with which it enters the cell (Kotte *et al.*, 2010). Also for yeast, it was occasionally proposed that it can measure metabolic flux (Ye *et al.*, 1999; Bisson & Kunathigan, 2003; Agrimi *et al.*, 2011), but it was similarly often explicitly stated that this would not be possible (Gamo *et al.*, 1994; Rodriguez & Flores, 2000; Youk & van Oudenaarden, 2009).

In this perspective article, we explore flux-sensing and flux-dependent regulation in yeast. Specifically, we will look (i) at what kind of evidence or indication we have for flux-dependent regulation, (ii) at general conceptual issues with sensing a rate and at potential flux-sensing mechanisms in yeast, and (iii) at some properties of the regulatory machinery that controls metabolism in a flux-dependent manner. As flux-sensing and flux-dependent regulation will not be accomplished via a single molecule, but rather will be realized by a whole system of molecules interacting in a very specific and likely complicated manner, systems biology approaches and modeling will definitely be required to generate a comprehensive understanding about this system. Thus, we conclude with a brief discussion about how systems biology could be instrumental towards this goal.

I. Evidence for metabolic flux-dependent regulation

An indication for flux-dependent regulation would be a correlation between the magnitude of a certain metabolic flux and a particular phenotype. Within limits, chemostat cultures allow modulation of metabolic fluxes, while at the same time keeping environmental conditions almost constant (Weusthuis *et al.*, 1994; Bull, 2010). A flux/phenotype correlation can be seen, for example, between the glucose uptake rates and the extent of usage of the fermentative pathway, with specifically particularly the ethanol excretion rate increasing with the glucose uptake rate. This is evidenced data from aerobic glucose-limited chemostat cultures of *S. cerevisiae* (cf. inset of Fig. 1A with data from Cortassaa & Aon, 1998; van Hoek *et al.*, 1998; Diderich *et al.*, 1999; Daran-Lapujade *et al.*, 2004; Frick & Wittmann, 2005; Daran-Lapujade *et al.*, 2007; Jouhten *et al.*, 2008; Basso *et al.*, 2010, cf. also Supplementary information).

In contrast to limiting the glucose uptake rate through environmental conditions, manipulating the maximal glucose uptake rate by genetic means is an alternative approach to assess the importance of the glucose uptake rate in dictating the metabolic mode. Elbing *et al.* (2004) constructed a set of hexose transporter mutant strains that only differ in their maximal glucose uptake rate (Elbing *et al.*, 2004). Similar to results obtained in glucose-limited chemostat cultures, it was found that the different transporter strains metabolize glucose to various extents by respiration and fermentation dependent on the rate of glucose uptake in an identical (i.e. high glucose) environment (Elbing *et al.*, 2004). This data perfectly aligns with the above mentioned chemostat data (cf. inset of Figure 1A and Supplementary information). For example, the phenotype of the strain with the lowest glucose uptake capability (TM6*) when grown at high glucose is the same as the one of the respective wildtype in the chemostat culture at a low dilution rate when the glucose influx is restricted by the environmental conditions. As in these experiments and in similar studies generalizing the findings from Elbing *et al.* to other *S. cerevisiae* strains (Reifenberger *et al.*, 1995; Ye *et al.*, 1999; Henricsson *et al.*, 2005), the environment was identical and the strains used differ only in their glucose transport capability, the glucose uptake rate seems to be the control factor for the metabolic mode and not environmental conditions such as the extracellular glucose concentration, which is inherently different in batch and

79 glucose-limited chemostat cultures. Indeed, also glucose sensing mutants switch from respiration to
80 fermentation at similar glucose uptake rates as their corresponding wildtype strain (cf. inset of Figure
81 1A with data from (Cortassaa & Aon, 1998). Also data from various different *S. cerevisiae* strains
82 grown in glucose batch cultures fit nicely to the so far presented data (cf. inset of Figure 1A with data
83 from Blom *et al.*, 2000; Peter Smits *et al.*, 2000; Elbing *et al.*, 2004; Otterstedt *et al.*, 2004; Blank *et*
84 *al.*, 2005; Cordier *et al.*, 2007; Velagapudi *et al.*, 2007; Heyland *et al.*, 2009; Kummel *et al.*, 2010;
85 Christen & Sauer, 2011; Costenoble *et al.*, 2011; Raab *et al.*, 2011).

86 As the correlation between glucose uptake rate and ethanol secretion rate seems to be independent of
87 the culturing methods (e.g. chemostat, batch cultures), the glucose concentration, and the S. *cerevisiae*
88 strains used, we set out to challenge this correlation further. In absence of oxygen yeasts are forced to
89 ferment, and this condition may uncouple glucose uptake rate from ethanol secretion rates. However,
90 even though under anaerobic conditions the ethanol production rate is generally higher as under
91 aerobic conditions (Verduyn *et al.*, 1990; Peter Smits *et al.*, 2000; Aguilera *et al.*, 2005; Jouhten *et al.*,
92 2008; Wiebe *et al.*, 2008), the anaerobic data fit well to the correlation presented in the inset of Figure
93 1A (cf. Figure 1A). Along the same line, data from yeast grown under various environmental
94 conditions, such as different pH or salinity of the medium (Heyland *et al.*, 2009), temperature (Tai *et*
95 *al.*, 2007; Postmus *et al.*, 2008; Heyland *et al.*, 2009), addition of weak organic acids (Larsson *et al.*,
96 1997; Abbott *et al.*, 2007; Daran-Lapujade *et al.*, 2007) and nitrogen limited chemostats (Larsson *et*
97 *al.*, 1997; Meijer *et al.*, 1998; Diderich *et al.*, 1999), also align well with the already plotted data (cf.
98 Figure 1A). Even when data from a wide range of *S. cerevisiae* mutant strains is added, the correlation
99 between glucose uptake rate and ethanol secretion rate still remains intact. Examples of such mutant
100 strains include strains with overexpressed enzymes of lower glycolysis (Peter Smits *et al.*, 2000),
101 uracil auxotrophies (Basso *et al.*, 2010), single deletions or overexpressions of various proteins
102 involved in glucose regulation (Blom *et al.*, 2000; Raab *et al.*, 2011) and carbon metabolism (Blank *et*
103 *al.*, 2005; Cordier *et al.*, 2007; Velagapudi *et al.*, 2007) (cf. Figure 1A).

104 Due to this robustness of the correlation between the glucose uptake and ethanol secretion rates, we
105 next asked whether this correlation would also be maintained in different yeast species. Yeast species

106 can roughly be subdivided in Crabtree positive and Crabtree negative yeast (De Deken, 1966).
107 Although the term "Crabtree effect" has been used with different meanings (Barford & Hall, 1979;
108 Van Urk *et al.*, 1990), 'Crabtree positive' means here that a certain yeast species can produce ethanol
109 under aerobic conditions, which 'Crabtree negative' cannot (De Deken, 1966; Verduyn *et al.*, 1992). It
110 is sometimes argued that Crabtree negative yeasts have a higher respiratory activity than Crabtree
111 positive yeasts and, as a result, Crabtree negative yeast would not have to resort to ethanol formation
112 under high glucose conditions (Verduyn *et al.*, 1991). However, when data from various Crabtree
113 positive and negative yeast species (gathered from Christen & Sauer, 2011; Rozpedowska *et al.*, 2011)
114 is plotted in Figure 1A, it turns out that the measured glucose uptake rates and ethanol secretion rates
115 fall perfectly on the already established correlation, indicating that Crabtree negative yeast species
116 have simply a lower glucose import rate and thus show no fermentative activity; in line of what was
117 also suggested earlier (Does & Bisson, 1989; van Urk *et al.*, 1989; Boles & Hollenberg, 1997;
118 Rozpedowska *et al.*, 2011). This idea is further supported by the observation that Crabtree negative
119 yeast species (*Candida utilis, Hansenula polymorpha* and *Kluyveromyces marxianus*) can ferment
120 under aerobic conditions by stimulating glucose uptake using a weak organic acid (Verduyn *et al.*,
121 1992) (data not shown).

122 Next we asked whether the correlation also holds when other sugars are taken up instead of glucose.
123 Generally, *S. cerevisiae* ferments glucose, but other sugars, such as sucrose, mannose and galactose,
124 are respired or fermented to different extents (Fendt & Sauer, 2010). Based on our established
125 correlation between glucose uptake and ethanol secretion rate, we asked whether the capability to
126 grow fermentatively on certain sugars is determined only by the respective import rate that yeast can
127 realize with a certain sugar. Indeed ethanol production rates obtained from *S. cerevisiae* grown on
128 galactose (Sierkstra *et al.*, 1993; Diderich *et al.*, 1999; Ostergaard *et al.*, 2000; Velagapudi *et al.*, 2007;
129 Costenoble *et al.*, 2011) compare well with those measured for glucose at the same sugar uptake rate
130 (see Figure 1A). This is also true for other sugars, such as fructose (Diderich *et al.*, 1999), maltose
131 (Weusthuis *et al.*, 1994; Wisselink *et al.*, 2007; de Kok *et al.*, 2011), arabinose (Wisselink *et al.*, 2007)
132 and sucrose (Basso *et al.*, 2010), when normalized to c-mols (see Figure 1A and Supplementary

information). Beyond, a relationship between import rate and the ability to ferment a particular sugar was also seen for maltotriose (Zastrow *et al.*, 2001; Dietvorst *et al.*, 2005) and cellobiose (Ha *et al.*, 2011). Identical to glucose, a strain with an engineered higher galactose uptake rate increased its respiro-fermentative metabolism with the ethanol production rate increasing linearly with glycolytic flux (Ostergaard *et al.*, 2000). Similarly, *Kluyveromyces lactis* switched to aerobic fermentation of galactose after introduction of the GAL2 gene (Goffrini *et al.*, 2002). In contrast, a yeast strain lacking invertase activity and with only a limited capacity to transport sucrose into the cell, showed a significantly reduced ethanol production rate (Badotti *et al.*, 2008). Overall, these observations indicate that the distribution between the respiratory and fermentative pathways is dependent not on the type of substrate being consumed but by the rate at which that substrate is imported.

The above mentioned observation that the ethanol excretion rate generally correlates with sugar uptake rate points towards a flux-dependent regulation. It could, however, also be conceivable that the growth rate determines the physiology, as, for example, was suggested by van Hoek *et al.*, 1998. To test this hypothesis, we plotted the ethanol excretion rates against the respective growth rates. As we here find absolutely no correlation (cf. Figure 1B), growth rate is very unlikely to be the determining control factor.

Another potential issue could be that the identified correlation could simply also be caused by a physical rate limitation somewhere in metabolism. Indeed it has been argued that the ethanol production is due to an overflow mechanism (Sonnleitner & Kappeli, 1986; van Hoek *et al.*, 1998; Zhuang *et al.*, 2011) meaning that there might a limitation in the TCA cycle or respiratory chain that causes the excretion of ethanol with increasing sugar uptake. If this would be the case, ethanol excretion would not be the result of an active, flux-dependent regulation. However, there are a number of indications that speak against the overflow hypothesis: (i) If the ethanol excretion would be the result of a capacity limitation in the respiratory chain, i.e. respiration reaches a maximum level at some point and no further NADH generated in the TCA cycle could be respired, then we would expect the oxygen uptake rate to stay at a constant (high) level with further increasing sugar uptake rates. As usually a decrease in the O_2 uptake rates is reported with increasing glucose uptake rates (Beck & von

160 Meyenburg, 1968; Verduyn *et al.*, 1992; van Hoek *et al.*, 1998; Canelas *et al.*, 2011), it seems that at
161 least respiration could not be the limiting factor. Further, the highest possible O_2 uptake rate can also
162 not be reached, which becomes obvious if we compare the O_2 uptake rate when yeast is grown on
163 ethanol with the highest O_2 uptake rate reported on when yeast grows on glucose (7.2 mmol
164 O_2/gDW/h in aerobic glucose chemostat culture at growth rates between 0.25 1/h and 0.33 1/h
165 (Canelas *et al.*, 2011) compared to 11.8-13 mmol O_2/gDW/h for unlimited growth on ethanol (as
166 estimated on the basis of measured ethanol uptake rates (Costenoble *et al.*, 2011) and flux variability
167 analysis (Mahadevan & Schilling, 2003)). (ii) It was reported that an alcohol dehydrogenase (*ADH1*)
168 deletion mutant in *S. cerevisiae* is very sick on high glucose conditions - a condition requiring
169 fermentation. In contrast, the *ADH1* deletion has no phenotype on galactose - a condition, on which *S.*
170 *cerevisiae* only respires. In turn, deletion of the TCA cycle isoenzyme succinate dehydrogenase
171 (*SDH1*) has no phenotype on glucose, but a lethal one on galactose (Ewald, Matt, Zamboni, 2011,
172 unpublished results). Similarly, blocking respiration on substrates that are typically respired leads to
173 no growth; a phenotype that can be rescued by overexpressing of the sugar transporters (Goffrini *et al.*,
174 2002; Fukuhara, 2003). Obviously, the sugar uptake rate and the metabolic mode need to fit together.
175 In our opinion, altogether these findings point more to a flux-dependent regulation than to a simple
176 physical overflow mechanism. If the latter would be true, then situations where the TCA cycle or the
177 respiration is blocked or reduced could simply be rescued by fermenting the galactose.

178 As a result, we propose that there is a universal regulatory system in place that (i) measures flux and
179 then (ii) determines the ratio in which glucose is utilized by respiration or fermentation according to an
180 evolutionary conserved program (see Figure 2A).

181 **II. How could metabolic flux be sensed?**

182 **a. Theoretical considerations**

183 We have seen that the global metabolic phenotype assumed by yeast is dependent on the substrate
184 uptake rate, i.e. that there is flux-dependent regulation. This implies that a flux signal needs to be

sensed within the cell. Before we discuss how a cell could actually achieve this, we first introduce – from a theoretical viewpoint – two concepts of flux-sensing.

A flux through a metabolic reaction is nothing else than a reaction rate. Here a rate *r* is defined as the ratio between an infinitesimally small change of a state quantity *x, dx,* and an infinitesimally small change in time *t, dt*, according to

$$r = \frac{dx}{dt} .$$

In other words, a rate is a derivative of a state variable. The first way to sense a rate *r,* is by measuring the difference of state *x* between two time points *t*,

$$r \approx \frac{\Delta x}{\Delta t} .$$

If the cell would follow this concept, it would need to have the capability to memorize two states, to measure time and to do mathematical operations in terms of subtractions and a division. It is obvious that cells cannot do this. However, there is a second possibility of how cells could get informed about a rate *r*. A rate *r* can be estimated from a state *x* (i.e. a metabolite concentration), if the functional dependencies of the system *f* are known, according to

$$r = f(x) .$$

This concept requires that the cell 'knows' the system that generates the state *x*, i.e. it has a model of the functional dependencies of the system, *f*. For cells to use this concept, it is important that a simple (ideally linear) dependency exists between *r* and *x* and that no other state variables influence this relationship as the controller (i.e. the regulatory system) that receives the flux-signal *x* needs to 'work' with this signal. Thus, cells that exploit flux-dependent regulation need to have a system that translates a flux (*r)* into a concentration of a biomolecule (*x)*. This, for example, flux-signaling metabolite *x* then would induce a flux-dependent regulation (see Figure 2B).

b. Hints towards flux-sensing mechanisms in yeast

Where in the metabolic network could the flux be sensed? The fact that the ethanol excretion rate not only correlates with the glucose uptake flux, but with all sugars' uptake rates (cf. Figure 1A) suggests that the flux sensor needs to be at a point in metabolism that is equally affected by all such sugars. The here mentioned sugars converge at either glucose 6-phopshate (G6P) (glucose, maltose, galactose), at fructose 6-phosphate (F6P) (fructose) or at both (sucrose). Thus, the flux sensing mechanism resides likely below F6P. On the basis of this reasoning, specific transporters or hexokinase 2 (Hxk2) can be excluded, although the latter is frequently suggested to be involved in flux-sensing (Bisson & Kunathigan, 2003).

If we argue here that eventually the flux is measured in glycolysis somewhere below F6P, and we had earlier only established a correlation between the sugar uptake rate and the global metabolic phenotype, then we need to check a correlation also exists between the sugar uptake rate and the glycolytic flux. A correlation is not necessarily expected, because glucose (or G6P) is also shuffled into the pentose phosphate pathway and into storage metabolism. Nevertheless, data from (partly ^{13}C based) metabolic flux analyses demonstrate that the glycolytic flux between G6P and F6P linearly correlates with the sugar uptake rate (see Figure 3 with data from Nissen *et al.*, 1997; Gombert *et al.*, 2001; Blank *et al.*, 2005; Frick & Wittmann, 2005; Jouhten *et al.*, 2008; Fendt & Sauer, 2010; Christen & Sauer, 2011) for glucose and galactose independent of the yeast species and the culture conditions employed and that this correlation is also robust against many environmental and genetic perturbations.

One way to establish a relationship of the kind $r=f(x)$ would be with x being the concentration of a metabolite that would correlate (ideally) linearly with the flux r. As metabolite concentrations were found to be highly specific for the limiting nutrient (Boer *et al.*, 2010), a signaling role for metabolites would not be so farfetched. Remarkably, the concentration of fructose-1,6 bisphosphate (FBP) seems to correlate with the sugar uptake rate when *S. cerevisiae* data from glucose batch (Fendt *et al.*, 2010) and glucose-limited chemostat cultures with different dilution rates (Canelas *et al.*, 2011) and different cultivation temperatures (Postmus *et al.*, 2008) are plotted (see Figure 4A). This correlation even holds for data from other Crabtree positive and negative yeast species (Christen & Sauer, 2011) (see Figure

4A). Thus, FBP could be a flux-signaling metabolite, like it was also suggested to be in *E. coli* (Kotte *et al.*, 2010). Interestingly, when the glucose concentration is suddenly increased in a glucose-limited chemostat culture or glucose is added to an ethanol-limited chemostat culture, the glucose influx rate increases with a concomitant increase in the concentration of FBP and onset of ethanol excretion. This indicates that FBP could not only report the flux in steady state, but also dynamically (Visser *et al.*, 2004; Bosch *et al.*, 2008).

In contrast, for example, the levels of ATP, ADP and AMP (obtained from van Meyenburg, 1969; Larsson *et al.*, 1997; Canelas *et al.*, 2011; Christen & Sauer, 2011), do not show any clear trend making it unlikely that the concentration of these metabolites would be the input for the respective regulatory machinery that controls the activity of the fermentative and respiratory pathways. This conclusion is further supported by findings from a recent study, in which mitochondrial NAD^+ carriers were deleted or overexpressed in *S. cerevisiae*. While these perturbations led to altered NAD and ATP levels, all the mutants switched from a fully respiratory metabolism to the respirofermentative one at the same glucose flux as the wild type (Vemuri *et al.*, 2007; Agrimi *et al.*, 2011), corroborating the idea that the levels of the energy cofactors levels are not likely to serve as flux signals.

How could the flux-information – imprinted into a metabolite's concentration (FBP, for example) – be coupled to the regulatory machinery to finally result in flux-dependent regulation? The first option for such coupling is an interaction of the flux-signaling metabolite directly with enzymes to activate or inactivate their activity (Figure 4), for which a couple of different mechanisms exist (Zorn & Wells, 2010). FBP, for example, is known to (i) activate pyruvate kinase (Otto *et al.*, 1986; Susan-Resiga & Nowak, 2003), (ii) have effects on the fructose 2,6-bisphosphate and AMP mediated activation of phosphofructokinase activity (Przybylski *et al.*, 1985) and (iii) inhibit oxidative phosphorylation through strongly inhibiting Complex IV (cytochrome c oxidase) and Complex III (ubiquinol:cytochrome c oxidoreductase) (Diaz-Ruiz *et al.*, 2008).

Alternatively, flux-signaling metabolites can be coupled to the regulatory machinery via interaction with signaling and regulatory proteins, such as kinases or transcription factors. Unlike in *E. coli*, for

which about a 100 metabolite-transcription factor interactions are known (ECOCYC database, Keseler *et al.*, 2011), we only know about a handful of such interactions in yeast, e.g. Gal3p, Put3p and Bas1p (for reviews see Sellick & Reece, 2005; Reece *et al.*, 2006; Campbell *et al.*, 2008). Here, the question is whether we simply do not know more (because they are hard to identify by classical biochemical means and as of today still no high-throughput method exists (Heinemann & Sauer, 2011) or whether they are not as prominent in yeast as they are in bacteria. Although most yeast transcription factors do not have small molecule binding pockets as, for example, the *E. coli* transcription factors (personal communication Nick Luscombe), novel metabolite-transcription factor interactions (Pinson *et al.*, 2009) and signaling protein interactions (Li & Snyder, 2011) were identified recently. Thus, there might be more to be discovered in the future. Similarly, we are also only about to realize that metabolite-binding RNA domains are also present in the genes of eukaryotes (Wachter, 2010), which might offer an alternative possibility how flux-signals imprinted into metabolite levels could be coupled to the regulatory machinery of a cell.

In contrast to sensing metabolic flux via flux-signaling metabolites, one could envision that certain enzymes directly sense metabolic flux. It is often speculated that hexokinase PII (Hxk2p) could do this, for example, via conformational changes that accompany the catalysis and that induce localization of Hxk2p to the nucleus or changes in a signaling complex of which Hxk2p is a component (Bisson & Kunathigan, 2003). All of this is however still elusive and as several sugars are not processed via hexokinase (such as galactose), a flux-sensor involving Hxk2 could not represent the full story.

III. Towards identifying the regulatory system that controls metabolism in flux-dependent manner

In response to a sensed glycolytic flux, a cell must be able to diverge the flux into either the respiratory or fermentative pathways. Due to the regulatory system's global and likely complex architecture - overarching multiple cellular levels - we have currently only a few fragmented pieces of evidence for what the actual regulatory system could be:

1. The system needs to be fast. Glucose pulses in glucose- and ethanol-limited chemostat cultures result in an immediate onset of ethanol excretion (Visser *et al.*, 2004; Bosch *et al.*, 2008), which indicates that at least part of the system must reside on the fast enzymatic level, e.g. in the form of metabolite-enzyme interactions. One such action could be the recently identified inhibition of the respiratory chain through FBP (Diaz-Ruiz *et al.*, 2008). Alternatively, fast metabolite-kinase interactions could also be involved in modulation of enzyme activity through phosphorylation. Here, several new enzyme phosphorylation sites were recently identified (Breitkreutz *et al.*, 2010). With regard to the fact that the system needs to be fast, it is interesting to note that older findings with *in vitro* systems constituted of a small subset of glycolytic enzymes in a continuous system were found to show different states depending on the glucose feed rate (Schellenberger *et al.*, 1980).

2. Redox metabolism has likely a limited role in the system. Perturbations in the NAD+ metabolism (which lead to changes in cellular NAD and ATP levels and affect growth rate) did not break the correlation between the sugar uptake and ethanol excretion rate (Agrimi *et al.*, 2011). Also increasing respiration or non-respiratory NADH oxidation in *S. cerevisiae* had only minor effects on the correlation between glycolytic flux and ethanol production rate (Vemuri *et al.*, 2007) (the data from these two references is displayed in Figure 1A, cf. supplementary information for details). Thus, although redox metabolism might be involved to a small extend in establishing overflow metabolism (Vemuri *et al.*, 2007), it does not seem to have a major influence on the distribution between respiratory and fermentative metabolism.

3. Genetic and chemical perturbations that block either the respiratory or the fermentative pathway result in complete system failures only at certain sugar uptake rates. For example, impinging on the capacity of the fermentative pathway by deletion of *ADH1* leads to a sick phenotype in glucose batch conditions (i.e. a high glucose uptake rate condition demanding fermentative metabolism), while this deletion has basically no phenotype on galactose (i.e. a low sugar uptake rate condition that demands a respiratory metabolism) (Ewald, Matt and Zamboni, 2011, unpublished). Vice versa, a deletion in the TCA cycle isoenzyme *SDH1*, which likely puts an upper limit on the flux through the TCA cycle, shows a practically lethal phenotype on galactose, while on glucose this mutation does not have a

phenotype (Ewald, Matt and Zamboni, 2011, unpublished). Along the same lines, blocking the respiratory chain with antimycin A only blocks growth in strains under conditions with a low sugar uptake rate (requiring a respiratory metabolism), while fermenting cells are not susceptible (Goffrini *et al.*, 2002; Fukuhara, 2003; Merico *et al.*, 2007). These observations suggest that when certain pathway cannot be used, because they are either chemically or genetically blocked, the regulatory system is not flexible enough to simply switch to the other metabolic mode.

Conclusion

Currently, we have only a very limited understanding about the sensing and regulation system that - in *S. cerevisiae* and also in other yeast species - is responsible for the distribution of flux into the respiratory or fermentative pathways. What we basically know today is the following: (i) The system seems to regulate the metabolic phenotype in a glycolytic flux-dependent manner, which is basically independent of yeast species, growth conditions, sugar source and many different genetic and environmental perturbations and thus seems to be very robust. (cf. section I). (ii) The concentration of FBP (and likely also other metabolites) is flux-dependent making them ideally suited as potential flux-sensors (cf. section II). (iii) There are only very fragmented insights into the regulatory system's characteristics (cf. section III).

To eventually obtain a complete systems-level understanding of how eventually 'metabolic flux controls metabolic flux', it is clear that next to the classical tools of biological research also various systems biology approaches (Heinemann & Sauer, 2010) will be required. For example, high-throughput analytical technologies such as metabolomics and fluxomics will be required for identification of further potentially existing flux-signaling metabolites, analytical procedures for detection of novel small molecule-protein interactions and phospho-proteomics to further investigate the relevance of enzyme phosphorylation. Next to these discovery-driven applications of these modern -omics technologies, it will also be required to generate molecule abundance data which after subjecting them to computational top-down analyses will likely be able to extract regulatory interactions . Finally, bottom-up modeling approaches will be required to test hypothesis on whether

certain behavior can emerge from the quantitative and dynamic interaction of a select set of molecular players. Here, the grand challenge will be to find the right level of abstraction for the model overarching multiple levels of the cell. Only with a model available, it will ultimately be possible to show whether, and if yes how metabolic flux is sensed and used for regulation.

Conclusion

To ultimately understand how the intricate system of the flux-sensing mechanism(s) and the respective regulatory machinery works, we will need to draw on all different branches of systems biology ranging from large-scale experimental approaches via top-down analyses to detailed modeling efforts.

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Figure legends

Figure 1: (A) Ethanol production rate as a function of sugar uptake rate (normalized to c-mol); **inset**: wild type *S. cerevisiae* on glucose batch cultures (black open squares); wildtype *S. cerevisiae* on glucose limited chemostat cultures (black open triangles); *S. cerevisiae* hexose transporter mutant strains on glucose batch cultures (black open diamonds), arrows indicate measurement values of the TM6* strain (see main text); *S. cerevisiae* glucose sensing mutant strains on glucose chemostat cultures (red circles); **main**: *S. cerevisiae* data from inset pooled (black squares); environmentally and genetically perturbed *S. cerevisiae* on glucose, incl. anaerobic conditions (blue triangles); *S. cerevisiae* wildtype and mutants on other sugars with different environmental conditions (red circles); other wildtype yeast species on glucose (green diamonds). **(B)** Ethanol production rate as a function of the growth rate; *S. cerevisiae* data from inset pooled (black squares); environmentally and genetically perturbed *S. cerevisiae* on glucose, incl. anaerobic conditions (blue triangles); *S. cerevisiae* wildtype and mutants on other sugars with different environmental conditions (red circles); other wildtype yeast species on glucose (green diamonds). In some cases, data was not available for both, the ethanol production rate and the growth rate. The references for the respective data points are provided in the supplement.

Figure 2: (A) The collected data in Fig. 1A suggest that there is a system (indicated by the black box) in place that depending on the sugar uptake rate generates different metabolic phenotypes in terms of fermentative and respiratory activity. **(B)** This system likely comprises of a flux-sensor that connects a rate r to a state x, which is received by a controller that in turn realizes the necessary regulatory adjustments in a flux-dependent manner.

Figure 3: Glucose isomerase flux as a function of the sugar uptake rate (normalized to c-mol); data from *S. cerevisiae* on glucose batch and chemostat cultures (black squares); environmentally and genetically perturbed *S. cerevisiae* on glucose (blue triangles); *S. cerevisiae* on galactose batch conditions (red circles); other yeast species on glucose batch conditions (green diamonds).

607 **Figure 4:** Metabolite levels as a function of sugar uptake rate (normalized to c-mols). **(A)** Fructose-
608 1,6-bisphosphate concentrations levels, **(B)** ATP concentration levels, **(C)** ADP concentration levels,
609 **(D)** AMP concentration levels. *S. cerevisiae* glucose batch and chemostat cultures (black squares), *S.*
610 *cerevisiae* environmental perturbation (blue triangles), other yeast species on glucose batch cultures
611 (green diamonds).

612 **Figure 5:** Different manners in which flux-information imprinted into concentration levels of flux-
613 signaling metabolites could be coupled to the regulatory machinery.

614